REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
14-02-2005	Final	27-07-2004 to 14-02-2005

4. TITLE AND SUBTITLE
Real Time Monitoring of Signaling Pathways in Biological Cells

5a. CONTRACT NUMBER
W31P4Q-04-C-R283

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Brogan, Louise, J, Cohen, Brian, D.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Evident Technologies

216 River Street
Suite 200
Troy, NY 12180

8. PERFORMING ORGANIZATION REPORT NUMBER
DARSB-JUL2704

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
US Army Aviation and Missle Command
ATTN: Thomas Bramhall
Bldg 7804 Rm 222
Restone Arsenal, AL 35893

10. SPONSOR/MONITOR'S ACRONYM(S)
AMSRD-AMR-WS-DP-SB

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
No limitations.

DISTRIBUTION STATEMENT A
Approved for Public Release
Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The goal of this research project was to determine the efficacy of EviTags as *in situ* nanoprobes. The experimental design used a fluorescence resonance energy transfer (FRET)-based approach to show how EviTags can monitor real-time cellular events, in particular, cell surface receptor trafficking and mRNA stability. EviTags were hypothesized to be excellent FRET donors when paired with organic dyes as acceptors because of their unique optical properties. The broad absorption spectra of EviTags allows for low wavelength excitation that does not overlap with organic dye excitation and consequently eliminates acceptor signal bleed through contamination. EviTags also have narrow full width-half max emission spectra with minimal, if any, spectral overlap with acceptor emission spectra thereby eliminating donor signal bleed through contamination. To evaluate EviTags as probes for cell surface receptor trafficking, the transferrin receptor was used as a model system. To address how EviTags can be used to measure mRNA stability, molecular beacon technology was employed. The results of the first phase of this project show EviTags to be highly efficient FRET donors, capable of being delivered into cells, and to be promising *in situ* nanoprobes.

15. SUBJECT TERMS
quantum dots, molecular beacons, fluorescence resonance energy transfer

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Louise J. Brogan, Ph.D.
U	U	U	UU	12	19b. TELEPHONE NUMBER *(include area code)* (518) 273-6266

Introduction:

The goal of this research project was to determine the efficacy of EviTags as *in situ* nanoprobes. The experimental design used a fluorescence resonance energy transfer (FRET)-based approach to show how EviTags can monitor real-time cellular events, in particular, cell surface receptor trafficking and mRNA stability. EviTags were hypothesized to be excellent FRET donors when paired with organic dyes as acceptors because of their unique optical properties. The broad absorption spectra of EviTags allows for low wavelength excitation that does not overlap with organic dye excitation and consequently eliminates acceptor signal bleed through contamination. EviTags also have narrow full width-half max emission spectra with minimal, if any, spectral overlap with acceptor emission spectra thereby eliminating donor signal bleed through contamination. To evaluate EviTags as probes for cell surface receptor trafficking, the transferrin receptor was used as a model system. To address how EviTags can be used to measure mRNA stability, molecular beacon technology was employed. The results of the first phase of this project show EviTags to be highly efficient FRET donors, capable of being delivered into cells, and to be promising *in situ* nanoprobes.

Cell Surface Receptor Trafficking. Cell surface receptors play an important role in controlling cell signaling events by mediating the transport of molecules in and out of the cells. Cell surface receptors also function to regulate intracellular activity through several mechanisms of molecular trafficking. The transferrin-transferrin receptor system mediates the internalization of iron into cells and is pivotal to the homeostasis of iron in living cells. The endocytic trafficking of transferrin-transferrin receptor complexes in non-polarized MDCK cells presents a good model biological system to evaluate EviTags as *in situ* nanoprobes as several trafficking pathways can be tracked. Transferrin-transferrin receptor complexes are internalized into sorting endosomes

via clathrin-coated pits from where they can be recycled back to the plasma membrane directly or via recycling endosomes. Transferrin receptor endocytic and recycling pathways can be affected by different regulatory pathways however the sorting machinery involved in the regulation of transferrin receptor transport steps has not yet been fully characterized. The objective of this research plan is to use the transferrin-transferrin receptor system to assay the ability of EviTags to transfer energy to organic acceptor molecules in the plasma membrane and endocytic membranes of non-polarized MDCK cells. The EviTag-based FRET assay was designed to provide information on the dynamics, distrubution and organization of the transferrin-transferrin receptor complexes in the plasma membrane and in sorting and recycling endosomes, as well as on their ability to interact with associated proteins in living cells. To achieve the overall objective of the assay design, we first evaluated the ability to deliver transferrin-EviTag conjugates into cells in a transferrin receptor mediated manner. Additionally, we tested the efficacy of EviTags as FRET donors upon uptake into cells. The results of these initial approaches are shown here and set the stage for the next phase of the research program.

mRNA Stability. Changes in mRNA expression reflect how a cell responds to stimuli. Being able to detect the effects of cellular treatments by measuring mRNA levels *in situ* and in live cells is important to many areas of biology from basic research to drug discovery. Traditional methods of measuring mRNA expression *e.g.*, northern blots and RT-PCR, are normally applied to mRNA extracted from the cellular context or to mRNA in fixed cells (*in situ* RT-PCR) and are not effective at revealing mRNA changes in live cells. In recent years, the use of fluorescent molecules as intracellular mRNA probes has expanded our detection capabilities by enabling researchers to utilize FRET as a reporter of intracellular activity.

FRET is the distance dependent radiationless transfer of energy from one fluorescent molecule (FRET donor) to another (FRET acceptor). A FRET donor:acceptor pair is selected based on overlapping donor emission and acceptor excitation spectra. An example of how FRET can be used to detect changes in the expression of a particular mRNA is with molecular beacons.

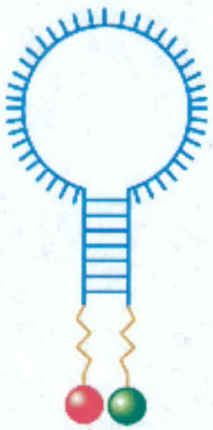

Figure 1. Molecular Beacon

Molecular Beacons are specially designed oligonucleotides conjugated at each end to different fluorophores (a FRET donor at one end, and a FRET acceptor at the other). As seen in figure 1, the oligonucleotide is designed to take on a specific structure in solution, commonly known as a stem-loop or hairpin. In this structure, a series of complimentary nucleotides base-pair as if they were on separate strands of DNA. The intervening sequence between the regions of hybridization is designed not to be included in the stem, but rather to remain single stranded, to form the loop part of the hairpin structure. The loop sequence is engineered to be specific for the gene (anti-sense) that is to be detected. The result of the base-pairing between the ends of the oligonucleotide is the association of the FRET donor and acceptor. When the molecular beacon is excited at the donor specific excitation wavelength, FRET occurs and donor emission is not detected, but acceptor emission is. When the beacons encounter the target mRNA molecule, the complimentary loop region of the beacon will base pair with that target. The interaction between the loop and the target molecule is strong enough to disrupt the base-pairing of the stem and consequently diminish the FRET signal. The loss of FRET can be observed as the donor fluorophore emission is restored.

The objectives for the first phase of this program were divided into these four tasks:

1) Investigate the placement of nanoprobes into biological cells to study signaling pathways.
2) Explore appropriate engineering and bio-conjugation of these probes to detect specific

molecular targets within cells in a non-intrusive manner.

3) Study and quantify data/signal acquisition from activation of specific molecular events within the cell.
4) Demonstrate feasibility of using nanoprobes for high SNR (Signal-to-Noise Ratio) real-time transduction of cellular signals.

The results presented in this report illustrate successful completion of these tasks with the exception of Task 3 which has been agreed to be pursued as part of the second phase of this research project.

Results and Discussion:

Task 1: Investigate the placement of nanoprobes into biological cells to study signaling pathways.

Cell Surface Receptor Trafficking. Transferrin-EviTag conjugates were added to transferrin receptor positive cells at relatively low concentrations (~25ug/ml) to investigate whether the nanoprobe could be placed into endocytic compartments in the cells and subsequently be used to reflect transferrin receptor activity. Hops Yellow (HY) EviTags were conjugated to the transferrin protein (using the EDC/s-NHS coupling protocol) and used to probe the transferrin receptor on non-polarized MDCK cells. Transferrin-EviTags were added to live MDCK cells in culture media for 30 minutes at 37degrees Celsius. The cells were then washed with PBS and fixed with 4% paraformaldehyde and mounted with glycerol. Confocal microscopy was used to image through the *z*-plane of a monolayer of cells to determine the overall efficacy of transferrin receptor mediated uptake of the transferrin-EviTag conjugate. The results of this assay show that Evitags can be delivered into cells in a receptor-mediated manner (Figure 2). The acquired confocal microscope images show intracellular uptake of the transferrin-Evitag conjugate in a pattern that is indicative of transferrin receptor mediated uptake. Evitags alone will not show this level of uptake when loaded onto cells at the same concentrations.

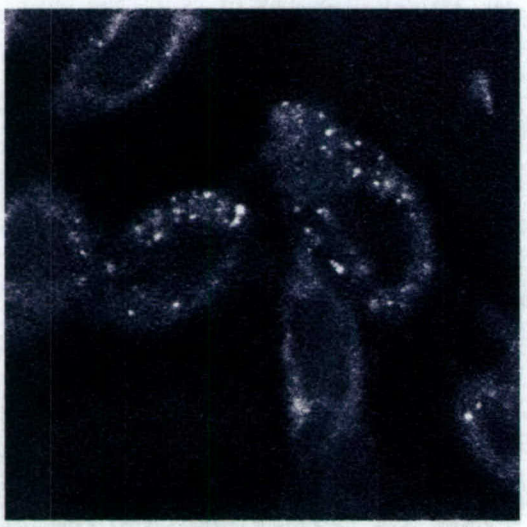

Figure 2. Transferrin receptor mediated uptake of transferrin-HY EviTag conjugates. Confocal image of MDCK cell cross-section

mRNA Stability. Cationic lipid-based transfection reagents were used for intracellular delivery of the EviTag molecular beacons. In three separate experiments with lipid transfection reagents (Lipofectamine Plus, Invitrogen) it was found that liposomes enhanced the transfection of the molecular beacons, as evidenced by an increase in fluorescence (Figure 3). To eliminate the possibility of signal bleed through contamination between the Adirondack Green EviTag and the HEX (hexachlorofluorescein) dye, the tunable filter detection window on the confocal microscope was set to 580nm. The overall transfection efficiency was variable therefore it will be important to select cells with the highest levels of transfected nanoprobe when assessing changes in target mRNA levels. Interestingly, three hours post-transfection no obvious change in morphology was observed suggesting minimal, if any, EviTag toxicity upon cellular transfection of the EviTag-molecular beacon nanoprobe.

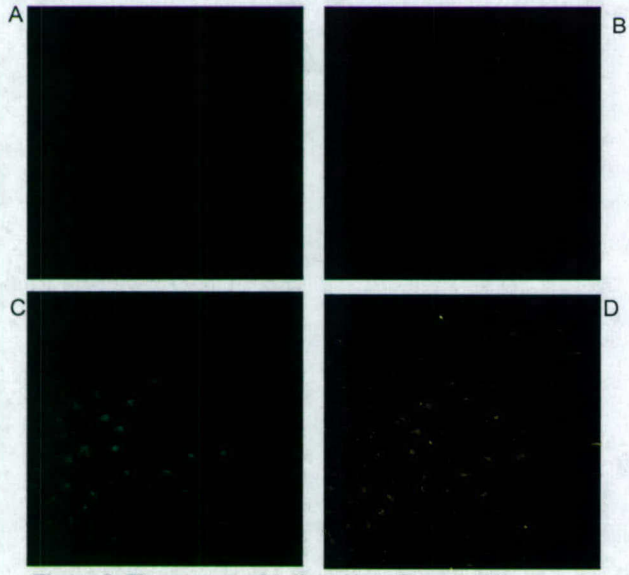

Figure 3. Fluoresence of Adirondack Green/HEX molecular beacons. A & B no transfection agent. C & D in the presence of Lipofectamine Plus. In A & C, fluorescence was detected at 540+/-10nm. In B & D, fluorescence was detected at 580nm+/-10nm. Images were taken with a Leica SP2 confocal microscope under identical exposure times.

Task 2: Explore appropriate engineering and bio-conjugation of these probes to detect specific molecular targets within cells in a non-intrusive manner.

Cell Surface Receptor Trafficking. EviTag surfaces were engineered to be terminated with functional groups to facilitate the coupling to specific ligands that can be used to address biological questions. The approach used in this study involved the bio-conjugation of the transferrin protein to carboxyl-functionalized EviTags. Specifically, Hops Yellow (peak emission 566nm) EviTags (molecular weight >100kD <300kD) were conjugated to the transferrin protein (molecular weight ~55kD) using EDC/sulfo-NHS coupling chemistry. The coupling protocol resulted in two fractions of transferrin-EviTag conjugates separated by molecular weight. One fraction was greater than 300kD and the other was less than 300kD but greater than 100kD. The high molecular weight fraction represents EviTags with a greater percent of transferrin molecules coupled to the surface. Each fraction was used in a receptor mediated uptake assay to identify the optimal ratio of transferrin to EviTag. Interestingly, both fractions showed similar uptake patterns suggesting that the degree of coupling is not the deciding factor for receptor interaction and that there may be other factors (e.g., sterric hindrance) that play a role. Overall, the surface functionality and conjugation method used to couple EviTags to the transferrin protein was appropriate and effective at generating a nanoprobe that can be used to detect molecular interactions within cells.

mRNA Stability. To minimize the radial distance between the quantum dot core of the EviTag and the organic dye (acceptor) for optimal FRET signaling, a proprietary EviTag surface coating material was developed. Figure 4 compares the overall fluorescence of the Adirondack Green EviTags with the original coating material (version 1) to Adirondack Green EviTags with the

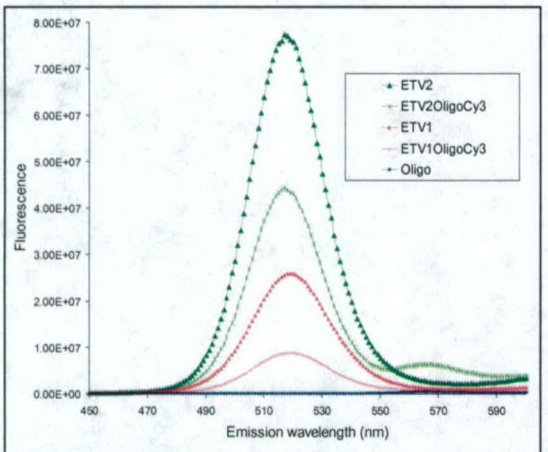

Figure 4. Comparison of version 1 EviTag (ETV1) to version 2 EviTag (ETV2) fluorescence.

new coating material (version 2). The new coating resulted in significantly stronger EviTag fluorescence emission and enhanced energy transfer in the molecular beacon-EviTag complex.

When coupled to Cy3 labeled-molecular beacons, the FRET signal is more significant with the version 2 Adirondack Green EviTags than with the version 1 Adirondack Green EviTags, the latter being nearly undetectable. The overall FRET signal is minimal, most likely due to the small spectral overlap between the EviTag emission and the Cy3 dye absorption. This observation led to the selection of the fluorescein derivative, HEX (hexachlorfluorescein) as a better acceptor for the Adirondack Green EviTags. The observed increase in FRET signaling to the HEX-acceptor is illustrated in Figure 5.

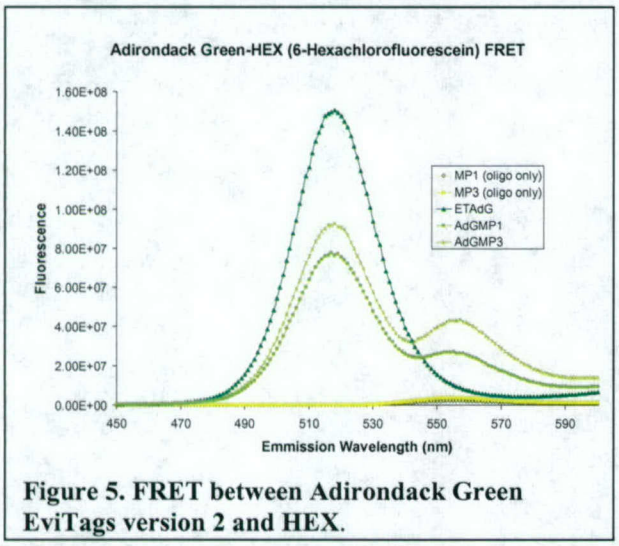

Figure 5. FRET between Adirondack Green EviTags version 2 and HEX.

To attain optimal bio-conjugation of the dye-labeled molecular beacon to the version 2 Evitags an alternative chemical approach to what was used with the version 1 EviTags was developed. Specifically, the heterobifunctional crosslinker sulfo-SMCC was used for linking the amine groups on the surface of the EviTag with the free sulfhydryl engineered onto the 5' end of the molecular beacon oligonucleotide. Optimal coupling of the acceptor dye-labeled molecular beacons to the EviTag surface is critical for efficient FRET signaling.

Task 3: Study and quantify data/signal acquisition from activation of specific molecular events within the cell.

This task was not addressed in this phase of the project and will be addressed in the second phase.

Task 4: Demonstrate feasibility of using nanoprobes for high SNR (Signal-to-Noise Ratio), real-time transduction of cellular signals.

Cell Surface Receptor Trafficking. We tested whether biotin-EviTags can act as FRET donors for streptavidin (SA)-labeled organic dye acceptors upon uptake into cells. Biotin-Hops Yellow (HY) EviTags were used as the FRET donor and SA-Alexa568 (Molecular Probes) was used as the FRET acceptor. These two molecules show a strong spectral overlap with no acceptor signal bleed through and reduced donor signal bleed through. One of the major advantages of using EviTags as FRET donors is the ability to select donor-acceptor pairs that show strong overlap with reduced or even non-significant signal bleed through. The HY-Alexa568 donor-acceptor pair would fit this objective when using the 458nm laser line as the donor excitation and filters that collect acceptor channel emission beyond ~600nm. Here, we used the 585LP emission filter, which was available in the Zeiss510 Meta confocal microscope used in this experiment. Biotin-HY/SA-Alexa568 complexes were incubated at high concnetration with non-polarized MDCK cells for 2 hours at 37C. Some of these complexes were internalized by fluid-phase endocytosis as suggested by the labeled punctate structures that are analogous to endocytic structures (see figure 6D).

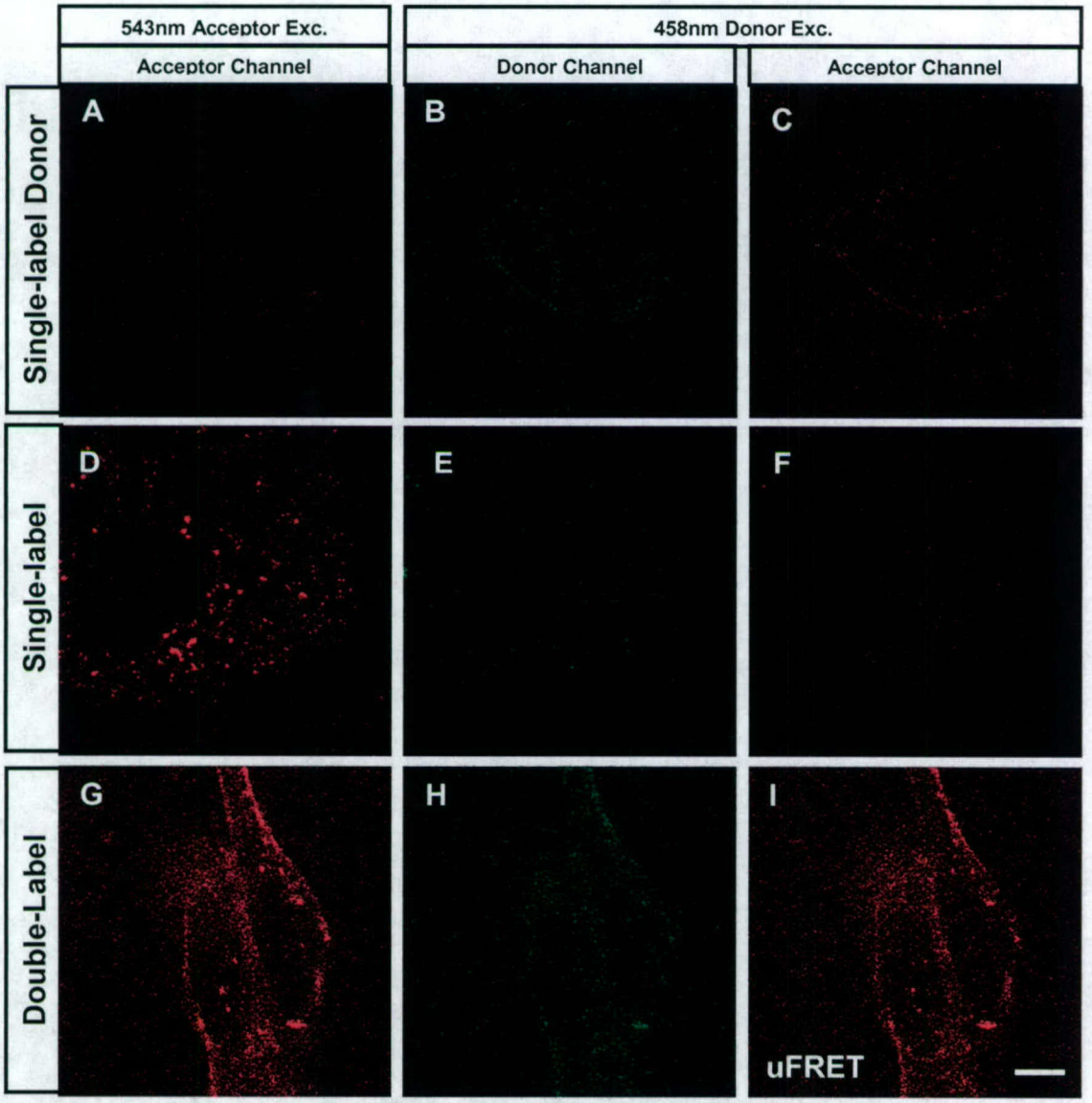

Figure 6. Confocal microscopy for FRET analysis using HY-biotin EviTags as donors.
(A) Fluorescence in the acceptor channel caused by 543nm excitation; **(B)** Unquenched donor (uD); **(C)** Donor signal bleedthrough, i.e., spillover of donor signal into the acceptor channel; **(D)** Fluoresc reference levels; **(E)** Fluorescence in the donor channel caused by 458nm excitation; **(F)** Acceptor si bleed through, i.e., fluorescence in the acceptor channel caused by donor excitation; **(G)** Fluorescenc reference levels; **(H)** Quenched donor; **(I)** Uncorrected FRET (uFRET), i.e., contains donor and acce signal bleed through. Bar = 10micron.

The nine images shown in Figure 6 were collected from donor and acceptor single-labeled and double-labeled cells using a Zeiss 510META confocal microscope with the following multi-tracking imaging conditions; 8-bit, 512x512 resolution, pinhole ~143um, 458nm donor and

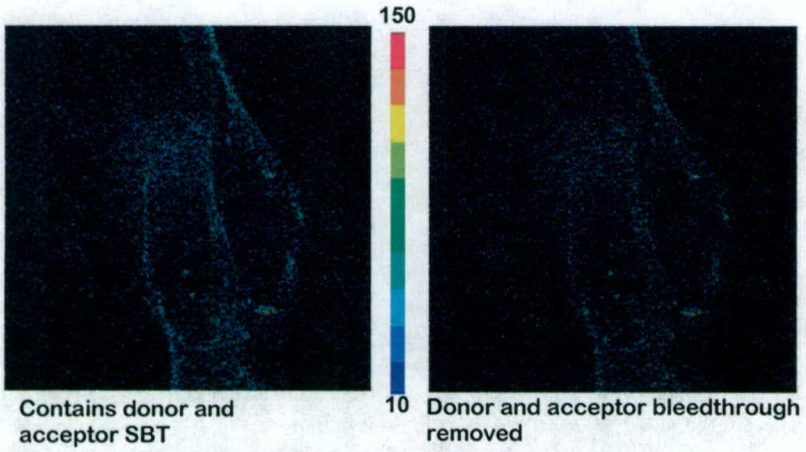

Figure 7. Extent of signal bleed through correction between uncorrected and processed FRET. Left, uFRET is the domor excitation/acceptor channel image; **Right,** the uFRET image after processing by the PFRET custom algorigthm (CircusSoft), which removes donor and acceptor signal bleed through.

543nm acceptor excitation, acceptor emission LP585 and donor emission LP505 filters; gain and black levels were kept constant during imaging.

Seven of those images (Figure 6B-D&F-I) were then processed to generate the corrected FRET image (Figure 7, right) that contains the actual energy transfer levels and to calculate the efficiency of energy transfer, E% (Figure 8). In this 'proof of principle' assay, we have shown that EviTags can act as FRET donors upon uptake into cells with a statistically determined E% of ~60% (Figure 8).

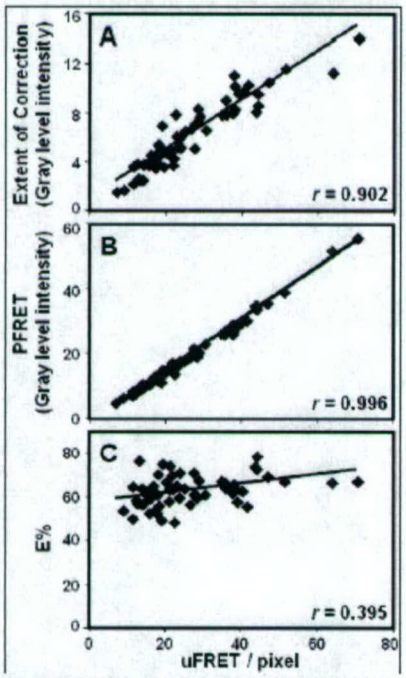

Figure 8. Comparison of uFRET with extent of correction (A), PFRET (or processed uFRET data) (B), and E% (C). r values show correlation levels.

mRNA Stability. The advantage of molecular beacons is the ability to detect specific nucleic acid molecules, either DNA or RNA by the hybridization of target molecules to the hairpin of the molecular beacon. This task was aimed at determining if hybridization of complementary oligonucleotides could be used to eliminate FRET between the quantum dot donor and the organic dye acceptor. As seen in Figure 9, addition of the target oligonucleotide was able to decrease in part, much of the energy transfer.

Salt concentrations, temperature, and target oligonucleotide concentrations were altered in attempts to enhance the overall inhibition or reversal of energy transfer however, little enhancement was observed. It is possible that optimal beacon 'melting' is not occurring with the specific target-loop sequence selected in this experiment. Since traditional molecular beacon approaches rely on release from quenching of the donor organic dye, it is possible that a portion of the beacons remain quenched but that proportion is swamped out by the increased signal. Regardless, we are able to achieve a significant reduction in the fluorescence of the organic dye and a significant increase in EviTag fluorescence, in the presence of the target oligonucleotide, therefore, this system has the sensitivity to detect a target sequence using a FRET-based approach.

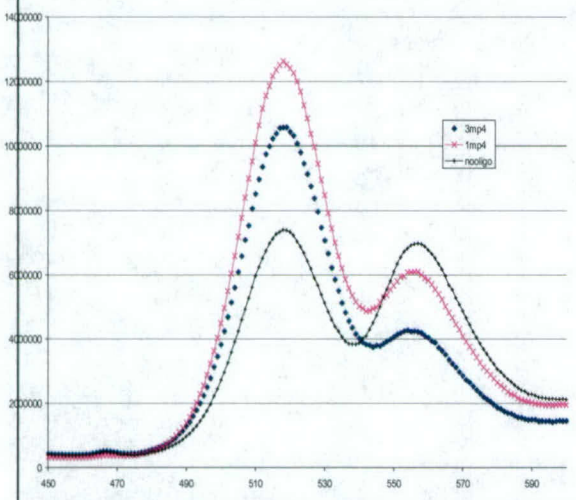

Figure 9. Reversal of FRET in an Evitag-oligonucleotide- organic dye molecular beacon. Molecular beacon probe was incubated with the complimentary oligonucleotide (MP4) at concentrations of 1mg/ml and 3mg/ml in solution. The sample was excited at 380nm to excite the EviTag and emission was measured from 450nm to 600nm. The peak centered at 520nm represents emission from the EviTag and the peak at 555nm is from the HEX dye. The decrease in emission from the HEX dye is mostly likely due to the hybridization of the complementary oligonucleotide disrupting the molecular beacon.